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Mutagenic Activities of Heterocyclic Amines in Chinese Hamster Lung Cells in Culture

by Masaaki Terada,* Minako Nagao,* Michie Nakayasu,* Hiromi Sakamoto,* Fumiko Nakasato,* and Takashi Sugimura*

A mutation assay system with Chinese hamster lung cells (CHL) using diphtheria toxin resistance as a selective marker has been established. The mutagenic activities of heterocyclic amines, originally isolated from pyrolyzates of amino acids and proteins, broiled fish and fried beef were assayed in cultured CHL cells in the absence and presence of a metabolic activation system, with diphtheria toxin resistance as a marker. All the heterocyclic amines tested except 3-amino-1,4-dimethyl-5*H*-pyrido [4,3-*b*]indole (Trp-P-1) required the presence of a metabolic activation system for mutagenicity on CHL cells. 3-Amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2) was the most mutagenic among the heterocyclic amines tested. Other compounds were also mutagenic in the following order of decreasing potency: Trp-P-1, 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-9*H*-pyrido[2,3-*b*]indole (AαC), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1) and 2-aminodipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-2).

Mutation testing using *Salmonella typhimurium* has successfully detected and identified mutagens in our daily foods. The application of a microbial mutation test for screening carcinogens is based on the reports showing overlapping of mutagenicity with carcinogenicity (*1*–*5*). Various mutagenic heterocyclic amines were isolated from pyrolyzates of amino acids, proteins, and cooked foods (*6,7*). All of these mutagenic heterocyclic amines, with which long-term animal carcinogenicity experiments have been completed, proved to be carcinogenic (*6,7*).

The mutagenicities of these compounds on mammalian cells have not been extensively studied. We have established a mutation assay system in Chinese hamster lung cells in culture, using diphtheria toxin resistance as a selective marker (*8*–*11*). The results of studies with this mutation assay system were summarized and presented with special emphasis on the mutagenic activities of heterocyclic amines (*9*). These compounds include 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1), 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2), 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1), 2-aminodipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-2), 2-amino-9*H*-pyrido[2,3-*b*]indole (AαC), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), and 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx).

*National Cancer Center Research Institute, 1-1, Tsukiji 5-chome, Chuo-ku, Tokyo 104, Japan.

Experimental

CHL cells were originally isolated from the lung tissue of a newborn Chinese hamster. The cells were maintained in Eagle's minimum essential medium supplemented with double concentrations of vitamins and amino acids (VAMEM) with 10% fetal bovine serum under the humidified atmosphere of 5% CO_2 in air at 37°C (*8*–*12*).

CHL cells were transferred to 5.5-cm^2 culture tubes with flat bottoms (A/S Nunc, Denmark). After 40 to 48 hr, the medium was changed to 1 mL of VAMEM with 3.5% fetal bovine serum or 1 mL of S9 mix medium, and the cells were cultured with a test compound for 3 hr in the CO_2 incubator at 37°C. The S9 mix medium consisted of 4.27 mM G6P, 0.75 mM NADP in VAMEM with 3.5% fetal bovine serum, and different concentrations of the S9 fraction from liver of rats treated with polychlorinated biphenyls. The protein concentration of the S9 fraction was about 35 mg/mL, and the optimum concentration of S9 was determined for each compound before assays. After exposure to a test compound, 2 to 4×10^5 cells were transferred after trypsinization to a 25-cm^2 flask containing 10 mL of VAMEM with 10% fetal bovine serum. After an expression time of 7 to 8 days, 2.5×10^5 cells were transferred to 100-mm dishes with VAMEM with 10% fetal bovine serum, containing 0.1 Lf/mL of diphtheria toxin. Simultaneously, 200 to 400 cells were transferred to 5 mL of toxin-free medium in 60-mm dishes for determination of plating efficiency.

After incubation for 7 to 8 days in medium in the presence and absence of the toxin, the cells were fixed with methanol and stained with Giemsa. Mutation frequency was expressed as the number of DT^r cells per 2.5×10^5 survivors. Concentrations of 0.01 to 1 Lf/mL diphtheria toxin were cytotoxic to CHL cells, and 0.1 Lf/mL diphtheria toxin was used to select DT^r cells. Under the assay conditions described, 7 to 9 days of expression periods were optimum for induction of DT^r cells.

The mutagenicities of alkylating agents, such as ethyl methanesulfonate (EMS), methyl methanesulfonate (MMS), methylnitrosourea (MNU), and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) were determined. With these compounds, there was a dose-dependent increase in the number of DT^r cells.

Properties of DT^r Cells

Diphtheria toxin is composed of an A fragment and a B fragment having molecular weights of 22,000 and 40,000, respectively. Fragment B attaches to receptors on the cells and fragment A enters the cells. Fragment A itself is an enzyme, which catalyzes ADP-ribosylation of elongation factor 2 at a unique modified amino acid, diphthamide (*13,14*). Once elongation factor 2 is ADP-ribosylated, the factor loses its activity and protein synthesis stops, leading to killing of the cells. There are at least two possible mechanisms by which the CHL cells are converted to DT^r (*15*). One involves a membrane alteration leading to reduced entry of the toxin into cells, and the other involves an alteration of elongation factor 2 such that ADP-ribosylation by the toxin does not occur. Seventeen DT^r clones were isolated in this work. Cytoplasmic fractions containing elongation factor 2 were prepared from the parental and mutant cells, and the toxin-catalyzed ADP-ribosylation of EF-2 was measured using nicotinamide-[U-^{14}C]adenine dinucleotide as a substrate (*8*). With the parental cells ADP-ribosylation increased linearly with the amounts of elongation factor 2, and 113 pmole of ADP-ribose per milligram protein of postribosomal supernatant was covalently linked to EF-2. By contrast, there was no detectable ADP-ribosylation of EF-2 from any of the 17 DT^r clones. These results showed that almost all the DT^r cells induced in the assay were due to alteration of elongation factor 2 itself. The high concentration of the toxin in the selection medium probably did not allow survival of variants with altered membrane permeability.

Mutagenic Activities of Heterocyclic Amines

The optimum concentration for S9 differs for each compound; for benzo(a)pyrene, the optimum concentration of S9 was 12.5 μL/mL of S9 mix, while for dimethylnitrosamine it was 100 μL/mL (*9*). Trp-P-1 showed mutagenic activity towards CHL cells in the absence of S9, and increasing the concentration of S9 resulted in reduced mutagenic activity. With Trp-P-2, the optimum concentration of S9 was 12 μL/mL, and there was a dose-dependent increase in the number of DT^r cells with increasing concentrations of Trp-P-2. The specific mutagenic activity of Trp-P-2 was the highest among the heterocyclic amines tested.

At the optimal concentration of S9 required for induction of DT^r cells by Glu-P-1 and Glu-P-2 (25 μL/mL) there was a dose-dependent increase in mutation frequency by both compounds. AαC also showed concentration-dependent mutagenicity with S9 optimized at 25 μL/mL. With IQ, MeIQ and MeIQx, the maximum number of DT^r cells was obtained with S9 concentration of 12 to 50 μL/mL. With S9 at 25 μL/mL, IQ, MeIQ, and MeIQx showed concentration-dependent mutagenicities. The cytotoxic effects of these compounds on CHL cells were much less than those of other heterocyclic amines tested.

With *S. typhimurium*, norharman and harman do not have mutagenic activity. But, their addition to certain nonmutagenic carcinogens results in marked mutagenicity in this bacterium. Norharman and harman are called comutagens (*16*). These compounds, however, were found to have mutagenic activities in CHL cells without S9 mix, using DT^r as a marker (*10*). The mutagenicities of these compounds decreased with increasing concentration of S9. The reasons for the difference in response to these compounds between CHL cells and the bacterial system remain to be clarified.

Comparison of Mutagenic Activities of Various Mutagen-Carcinogens

Mutation assay with the use of *S. typhimurium* has been widely and successfully employed to monitor and screen for mutagen-carcinogens in our daily life. Bacteria, however, differ from mammalian cells in the organization of genetic material, metabolism, transport, and DNA repair processes. In addition, the *S. typhimurium* assay measures reverse mutation, while the DT^r assay with CHL cells measures forward mutation. We have measured the mutagenicities of various compounds on CHL cells. For the comparison, the specific mutagenic activities were calculated from the linear portions of the concentration–response curve at optimal S9 concentration (when required), and are presented in Table 1. Nitropyrenes are among the most mutagenic compounds on *S. typhimurium* TA 98 without S9 mix (*17*). These compounds, ubiquitous in our environment, are produced by internal combustion engines and coal-fired power plants. They were also found in chicken grilled with sauce (*18*). The 1,6- and 1,8-dinitropyrenes without S9 mix were highly mutagenic to CHL cells, 1,3-dinitropyrene and 1,3,6-trinitropyrene being less mutagenic (*8*). No mutagenic activity was detected using 1-nitropyrene and 1,3,6,8-tetranitropyrene. The mutagenic activity of 1,6- and 1,8-dinitropyrenes was comparable to that of 4-nitroquinoline 1-oxide (4NQO). It should be noted that 1,3-, 1,6-, and 1,8-dinitropyrenes were carcinogenic when injected subcutaneously into

Table 1. Specific mutagenic activities of various chemicals to CHL cells.

	DT^r mutants per 10^6 survivors per μg[a]
Compounds not requiring S9 mix	
MNNG	4,000
4NQO	952
1,6-Dinitropyrene	720
1,8-Dinitropyrene	520
1,3-Dinitropyrene	256
1,3,6-Trinitropyrene	192
Trp-P-1	33
MMS	25
MNU	18
Harman	4.7
AF-2	4.4
Quercetin	2.0
Norharman	1.7
EMS	0.6
Coffee	0.2
Compounds requiring S9 mix	
Trp-P-2	160
BP	90
IQ	40
MeIQ	38
AαC	20
MeIQx	5.7
DMNA	4.0
Glu-P-1	1.2
Glu-P-2	0.3

[a] The specific mutagenic activity of each compound to CHL cells is expressed as DT^r cells per 10^6 survivors induced by each compound at the concentration of 1 μg/mL for 3 hr.

mice or rats, while 1-nitropyrene failed to induce tumors upon subcutaneous administration to rats (*19,20*).

Among the heterocyclic amines tested, Trp-P-2 was the most mutagenic, followed in order by IQ, MeIQ, Trp-P-1, AαC, MeIQx, Glu-P-1, and Glu-P-2. The order of the specific mutagenic activities of the compounds toward CHL cells differed from that of their activities toward *S. typhimurium*. It should be noted that mutagenic activity of crude material such as coffee could be measured with this assay system (*11*). The mutation assay with CHL cells, using diphtheria toxin resistance gives relatively reproducible results and can be recommended for use as a mammalian mutation method.

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